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CHEMOTACTIC PROPERTIES OF BRUCELLA SUIS

A STUDY OF PHAGOCYTOSIS OF BRUCELLA IN VITRO BY NORMAL, NONIMMUNE HUMAN LEUKOCYTES *

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Although human brucellosis has been subjected to widespread and comprehensive clinical investigations, the details of the pathogenesis of the disease are little understood. Sharp,[1] in a recent summary of reports of investigations of this disease, makes this statement: "A more or less definite type of infectious granuloma is suggested, the most striking feature of which is a nodular lesion resembling the tubercle." From our own observations on the experimental disease [2-6] we are able to confirm Sharp's view in regard to the granulomatous nature of the basic pathological process in brucellosis, but we have not been impressed by any striking resemblance of the lesions to those of tuberculosis. On the other hand, from a review of the very few carefully studied fatal cases of brucellosis that are available, we have been impressed by a resemblance of the lesions to those of Hodgkin's disease.[7] That the latter resemblance may have some particular significance has been suggested by the observations recently reported from this laboratory [3,7-9] that brucella can be isolated from cases of Hodgkin's disease with impressive frequency. It is with the hope of learning more of the pathogenesis of brucellosis and thereby studying further the possible relationship between this disease and Hodgkin's disease that the following investigation of the influence of brucella over leukocytes from the peripheral blood was undertaken.

One of the first things that must be done in the study of the pathogenesis of any infectious disease obviously is to determine what reaction occurs immediately following the introduction of the organism concerned into the tissues. As a general proposition, we know that an immediate local accumulation of phagocytic cells is usually the first

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thing that takes place. The general subject of phagocytosis in relation to infection and immunity has been of great interest since the work of Metchnikoff.[10] He thought that the polymorphonuclear neutrophils were the chief, if not the sole, agents responsible for resistance to bacterial invasion and that the failure of these elements to perform their phagocytic function would result in destruction of the host by the invader. Subsequently other workers [11-14] have adopted a somewhat different view, pointing to the fact that the circulating monocytes and especially the tissue macrophages appear to be the principal defenders of the body. Freedlander and Toomey,[14] for example, have shown that subcutaneously injected staphylococci are phagocytized by both types of cells, but are destroyed chiefly by macrophages. Likewise, Lucké, Strumia, Mudd, McCutcheon and Mudd [15] found that the degree of phagocytosis *in vitro* by the macrophages (including the monocytes) is approximately the same as that of the polymorphonuclear neutrophils. Furthermore, they and others have shown that while, in the experimental animal, the initial response to certain organisms (for example, the tubercle bacillus) is nearly the same for the cells of both types, in the later stages of development of the initial lesion, the monocytes and the macrophages always play the more important rôle.

If one takes into proper consideration these and other well known examples of the development of the initial cellular response to invading bacteria, it may be suspected that the characteristic features of brucellosis may depend to a great extent on the manner in which the phagocytic elements of the body react to brucella. A study of the phagocytosis of brucella *in vitro* by the white cells of the peripheral blood therefore appeared to us to be a logical point of departure for the study of this problem.

Experiments

Experimental Materials. In these experiments, Huddleson's strain 47 of *Brucella suis* from a 48-hour blood agar slant culture was employed; a nonhemolytic *Staphylococcus aureus* cultured in the same manner was chosen for comparative study. Whole human blood obtained by finger puncture with a capillary pipette was used as a source of leukocytes. For supravital studies, preparations were made with neutral red, pinacyanole and a combination of these two dyes (10 drops of neutral red and/or 10 drops of pinacyanole stock solution in 10 cc. of absolute alcohol) spread and dried in a film on the slide. Fixed preparations were stained with the following dyes: Wright's stain, hematoxylin and eosin, Kingsley's stain [16] and Giemsa's stain. Preparations for study were made according to slide-coverslip and hanging-drop methods as described below.

Experiment 1. Supravital Studies of Phagocytosis

A small amount of a saline suspension of live brucella was spread on a slide and upon this a small drop of blood was quickly placed; the preparation was then covered with a coverslip and sealed with a paraffin-vaseline mixture. Similar preparations were made using the staphylococcus. These preparations, on a warm stage in an incubator of 37° C., were observed for as long as 72 hours. Preparations supravitally stained with neutral red and/or pinacyanole also were made according to this technic. The leukocytes proved to be well differentiated in these preparations. The staphylococcus was readily identified both within and outside the cells, but brucella could not be identified except when the organisms were clumped; this was due to the small size of the organisms and to the confusion produced by particles in the plasma or by granules normally present in the cells. Smears of these same preparations were made by sliding the coverslip quickly off the slide; these were allowed to dry, after which the paraffin-vaseline was removed with xylene. These smears were stained according to a variety of methods as indicated above.

Experiment 2. Phagocytosis in Hanging-drop Preparations

Hanging-drop preparations for the study of phagocytosis of killed organisms were made in the following manner: A minute drop of distilled water suspension of organisms was placed in the center of a coverslip, allowed to dry and then gently heated for just a few seconds over a flame. This technic resulted in the production of a small ring of dead organisms on the coverslip. A drop of fresh whole blood was then allowed to clot over the organisms on the coverslip; following this, the coverslip was inverted over a hollow slide and sealed with paraffin-vaseline mixture. Preparations made in this manner were placed in the incubator and observed during periods varying from ½ to 72 hours. Because of difficulty in illuminating the central area of the hanging-drop of blood, observations on these preparations were restricted to the edge of the drop, quite beyond the ring formed by the concentration of organisms. This proved to be an unsatisfactory preparation and it was decided to apply Berman's method,[17] which consists of stripping the clotted drop away from the coverslip with filter paper and fixing and staining the remaining cells. Unfortunately, this usually produced a serious disturbance in the original arrangement of the cells. In further experiments it was determined that satisfactory preparations for study of the cells after incubation could be made by allowing the drop of blood to dry completely in the air, rinsing the dried blood with distilled water to dissolve the hemoglobin, and then drying again in the air. In

preparations of this type, the cells are killed quickly, are not disturbed in their arrangement and, when stained, are easily seen. Numerous stains were applied to these preparations, including Wright's, hematoxylin and eosin, and Kingsley's.[16] Brucella was not well stained by any of these; the staphylococcus stained very well with Kingsley's stain. The Giemsa staining method used by Wolbach in the study of *Rickettsia prowazeki*[18] was employed with excellent results.

Hanging-drop preparations for the study of phagocytosis of living organisms were made as just described except for omission of heating the dried suspension of organisms on the coverslip. (Drying for a short time does not kill all the organisms, as shown by cultures made from the coverslips.)

Control Preparations

Controls for the slide-coverslip method were made, using the same technic and vital stains but with no organisms; these were observed concurrently with the preparation of brucella and staphylococcus.

Controls for the hanging-drop method were made by touching sterile blood agar slants with the loop in the same manner as in making the suspension of organisms and duplicating each succeeding step.

Control of the phagocytic ability of the white blood cells of the blood employed under the experimental conditions was obtained through observation of the action of the whole blood cells toward the staphylococcus used for comparative study.

Experimental Observations

Living white blood cells were studied by the slide-coverslip method, both with and without supravital stains. In many of these preparations there were areas containing clumps of organisms and fluid, but no red cells. In a short time, these areas were invaded by polymorphonuclear neutrophils which rapidly cut swathes through the clumps of organisms, reaching out their pseudopodia and ingesting all of the organisms with which they came in contact. These pseudopodia conformed in shape exactly to the shape of the clumps of organisms and left no organisms behind when they withdrew. This process was observed to continue until the leukocytes were completely filled with organisms.

In the supravitally stained preparations, the staphylococci were seen clearly as unstained refractile bodies inside reddish stained vacuoles in the cytoplasm. The vacuoles were seen to increase in size, but the color became less intense as the cells gradually lost their vitality; after the cells died the vacuoles became colorless. The same type of vacuolization was seen in the preparation made with brucella following phagocytosis of these organisms, but the organisms could not be identified

inside the cells. In the control preparations without organisms this vacuolization began much later and was not nearly so massive. Neither staphylococcus nor brucella, dead or alive, regardless of their location within or outside the cells, was stained by the vital dyes. The organisms were not observed to grow in these preparations, perhaps because of exclusion of the air. No phagocytosis by cells other than the polymorphonuclear leukocytes was seen in these preparations. These cells were active for only a few hours; in contrast, in the unstained preparations they were active for a maximum of about 20 hours, after which they degenerated. In the unstained preparations, after about 20 hours the monocytes and lymphocytes exhibited active motion, which lasted as long as 60 to 70 hours. Even in the active state, these cells did not phagocytize either organisms or other cells. In the supravital preparations, death of these cells occurred within about the same time as that of the polymorphonuclear leukocytes.

In preparations supravitally stained with pinacyanole, phagocytosis by polymorphonuclear cells could be followed with ease. When cells are stained with this dye, the nuclei are dark purple and the cytoplasm light purple; mitochondria are also stained, as are some of the granules. The cells advance a clear layer of ectoplasm which contains no granules; from this many short finger-like processes extend. The ectoplasm first flows around an organism much as water flows along a surface and surrounds an object; next, the granular cytoplasm flows over the organism, which then moves with the rest of the streaming granules. Cells of other types move in a similar manner, but they were not seen to ingest the organisms.

In every hanging-drop preparation of either staphylococcus or brucella, dead or alive, observed from ½ hour incubation upward (with the exception of those in which the organisms were heated beyond a certain point), the neutrophilic polymorphonuclear cells in great numbers migrated to the ring of organisms on the coverslip (Fig. 1) and phagocytized numerous organisms (Fig. 2). The original distribution of the other white cells was not changed. In a few monocytes and eosinophils there were seen one or two organisms (these may have been superimposed), but certainly no marked phagocytosis by these cells was seen in any preparation. Control preparations made without organisms but with drops of distilled water which had been brought into contact with sterile blood agar showed no migration of cells.

When the organisms on the coverslips in the hanging-drop preparations were heated longer than a few seconds (not charred) they did not attract any polymorphonuclear leukocytes, were not phagocytized, and did not stain as they did in other preparations. Hanging-drop preparations made by drying without heating the organisms on the coverslip

showed, after incubation, that the organisms were still alive and had multiplied. In 12 hours, the polymorphonuclear leukocytes at the extreme periphery of the ring of organisms were completely filled with bacteria and some were surrounded by a small colony of organisms (Fig. 3). Comparing these preparations with the gently heated preparations it seemed that the organisms ingested by the polymorphonuclear cells had multiplied within these cells. (It was shown in the slide-coverslip preparations that the cells with ingested organisms are able to live as long as 20 hours.)

COMMENT

From the studies reported here, it appears that brucella offers little attraction *in vitro* for any of the normal circulating white blood cells except the neutrophilic polymorphonuclear leukocytes. This observation appears to be out of harmony with the general principle relating to phagocytosis as stated by Lucké and his associates,[15] namely, that "phagocytosis promoting properties of tropins apply similarly to macrophages and to polymorphonuclear leukocytes" and that "the mechanism of bacteriotropin action is the same for both kinds of cells." An explanation of this failure of brucella to attract normal monocytes is highly desirable in view of the fact that observations on the phagocytic activity of the tissue wandering cells—the tissue macrophages or clasmatocytes—in experimental brucellosis [3,5,19] have shown that these cells are commonly engorged with organisms. These latter observations are so impressive that it is suggested that brucella, at least, may propagate best in an intracellular environment. This apparent difference between the action of clasmatocytes and monocytes toward brucella may be a significant one, possibly indicating a fundamental difference between these cells. Such a distinction has been emphasized by Sabin and Doan [20] in their studies of the rôles played by the monocytes and the clasmatocytes in the development of the lesion in experimental tuberculosis. These workers suggest that phagocytosis is carried out chiefly by the clasmatocytes, whereas the monocytes form the tubercle in response to the irritating residue originating in the digestion of the tubercle bacilli by the clasmatocytes. (It is not illogical to postulate the operation of some such mechanism in the development of the brucella lesion and that this may lead to the development of cytological reactions such as those which characterize Hodgkin's disease.[21]) The problem obviously requires further study since in these preliminary experiments there are numerous factors as yet uncontrolled. The artificial conditions of the experiment doubtless were of some importance; this is suggested by the failure of the

staphylococci to attract the monocytes. Perhaps the properties of the particular strains of organisms used also may have been determining factors. Lastly, surely the concentration in the drop of blood of those immune bodies that usually influence the phagocytic activity of the leukocytes was material to the outcome of the experiments. A study of these and other factors concerned are problems for future investigation.

SUMMARY

1. The behavior of white blood cells from the circulating blood of a normal nonimmune person toward *Brucella suis* (Huddleson's strain 47) was studied *in vitro* in slide-coverslip and hanging-drop preparations using fixed and supravital staining technics.

2. In the above described preparations, *Br. suis* provoked an immediate response on the part of the neutrophilic polymorphonuclear leukocytes and was phagocytized quickly by these cells; this response was not exhibited by any of the other leukocytes.

3. When ingested by a polymorphonuclear leukocyte, brucella was found to be surrounded by a vacuole. The ingested organism was not necessarily killed by the cell; in some instances it appeared to multiply within the cell body.

4. Brucella loses some of its staining properties and its chemotactic effects upon the polymorphonuclear leukocytes when it is heated beyond a certain point.

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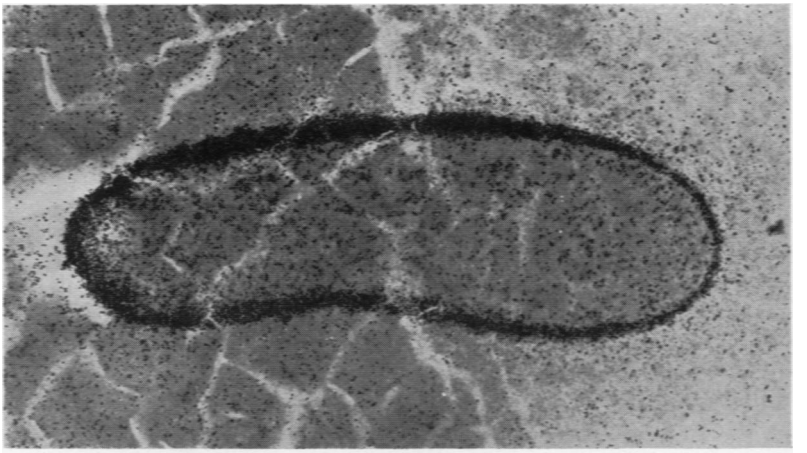
DESCRIPTION OF PLATE

PLATE 32

FIG. 1. Hanging-drop preparation of brucella and nonimmune human whole blood, showing a ring consisting of polymorphonuclear neutrophils which have migrated to and ingested large numbers of organisms previously placed on the coverslip and killed by heating gently. Incubated 1 hour. Giemsa's stain. About × 40.

FIG. 2. A small segment of the periphery of the ring illustrated in Figure 1. The black nuclei of the polymorphonuclear neutrophils are shown, surrounded by cytoplasm packed with large numbers of organisms. The edge of the ring of cells is sharply defined; this outlines exactly the area containing organisms. About × 1100.

FIG. 3. A preparation similar to that of Figure 1, except that the organisms, brucella, were not heated and therefore many were not killed. Incubated 12 hours. The dark areas are masses of organisms which have grown during the incubation of the preparation. The arrow points to a colony of organisms surrounding an engorged polymorphonuclear neutrophil which has migrated from the ring carrying organisms with it. About × 40.



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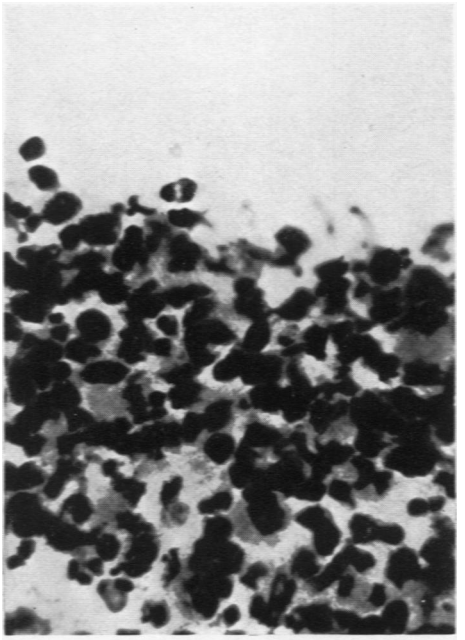

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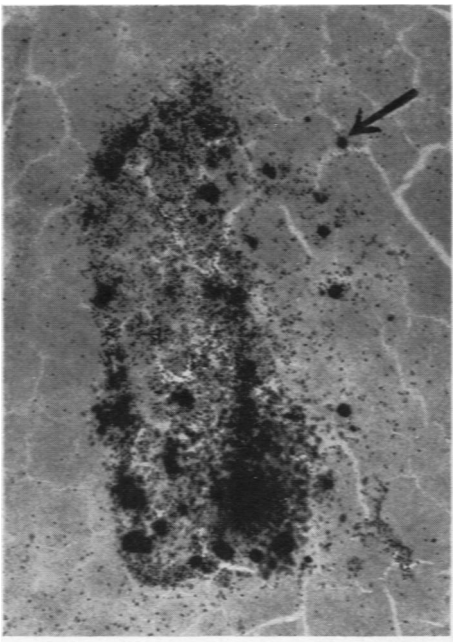

3

Dickey and Forbus

Chemotactic Properties of *Brucella suis*